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THE JOURNAL OF PARASITOLOGY
Vol. 62, No. 2, April 1976, p. 199–202

FORMATION OF SULFHYDRYL GROUPS IN THE WALLS OF *EIMERIA STIEDAI* AND *E. TENELLA* OOCYSTS SUBJECTED TO IN VITRO EXCYSTATION*

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ABSTRACT: *Eimeria stiedai* or *Eimeria tenella* oocysts were incubated in aqueous cysteine hydrochloride (cysHCl) under carbon dioxide (CO_2), aqueous cysHCl under air, water under CO_2 or water under air, and analyzed for sulfhydryl (–SH) groups. The cysHCl–CO_2 treatment produced more –SH groups than the other treatments and was effective in allowing activation of intact and sodium hypochlorite (NaOCl)-treated *E. stiedai* oocysts as well as NaOCl-treated *E. tenella* oocysts. The CO_2–cysHCl complex may act directly on the oocyst wall, especially in the micropylar region, to unmask lipid-shielded disulfide bridges, which are reduced to –SH groups. The reduction apparently disturbs the protein superstructure of the oocyst wall, promotes opening of the micropyle, and changes the impermeable state of the sporulated oocyst.

The precise mechanism of coccidian excystation has not been explained, although much information about the process has been gained in previous studies (Jackson, 1962; Hibbert and Hammond, 1968; Nyberg et al., 1968; Ryley, 1973). In vitro, oocyst walls must be mechanically or chemically altered, after which viable sporozoites can be stimulated to become motile and to excyst by exposure to a trypsin–bile solution. The walls of oocysts can be chemically altered by incubation in a reducing solution which has been flushed with carbon dioxide. The biochemical mechanism of the CO_2-reducing agent treatment is believed by some to be enzymatic, wherein the CO_2 activates an enzyme precursor located in the sporozoite (Hibbert and Hammond, 1968) or in the oocyst residual body (Ryley, 1973). Since an excystation-mediating enzyme has not been isolated or otherwise directly identified (Jolley and Nyberg, 1974), and since the oocyst wall has been shown to consist mainly of protein and lipoprotein (Monné and Hönig, 1954; Ryley, 1973), we undertook this study to investigate a possible direct effect of the CO_2-reducing agent complex on the oocyst walls of *Eimeria stiedai* and *E. tenella*.

Received for publication 7 October 1975.
* This study was partially supported by NSF Grants GB 8295 and GB 27425.
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MATERIALS AND METHODS

Oocysts were separated from fresh feces by standard screening, washing, and sucrose flotation techniques. When required, fine debris was removed by repeatedly washing the oocyst suspensions with 0.85% saline, followed by gradient centrifugation in 1:1 water:sucrose continuous gradient tubes. Cleaned oocysts were sporulated in 2.5% potassium dichromate through which air was bubbled for 5 days at room temperature. Sporulated oocysts were stored in the dichromate solution at 4 C until used, whereupon the dichromate was washed out with distilled water.

Oocyst walls were chemically altered by incubating sporulated oocysts in a shallow solution of aqueous 0.02 M cysteine hydrochloride (cysHCl) under an atmosphere of CO_2. The temperature and duration of incubation were 37 to 39 C for 3 to 4 hr with *E. stiedai* and 39 to 41 C for 10 to 16 hr with *E. tenella*. In all incubated suspensions, small samples of the oocysts were microscopically examined for oocyst wall warpage at 1- or 2-hr intervals to ascertain the progress of wall alteration; in addition, small samples were exposed at 37 to 40 C to a solution of 1% trypsin and 5% bile (pH 8) prepared with standard tris or phosphate buffers. The per cent of the oocyst alteration and sporozoite activation was determined by counting the average number of oocysts "activated" by the trypsin–bile exposure per 100 oocysts viewed. All tests performed on intact oocysts were also performed on oocysts whose outer wall layers had been removed with 2.5% sodium hypochlorite (NaOCl, Clorox®). For control, oocyst suspensions were incubated in distilled water under air, in distilled water under CO_2, or in aqueous 0.02 M cysHCl under air. Each complete set of test and control incubations were run simultaneously for a period of time sufficient to allow the test oocysts

TABLE I. *Sulfhydryl group equivalents (micromoles) on intact or sodium hypochlorite- treated oocysts incubated in water under air, water under CO_2, 0.02 M cysHCl under air, or 0.02 M cysHCl under CO_2.*

Species	Number of oocysts ($\times 10^8$)	Treatments			
		Air H_2O	CO_2 H_2O	Air cysHCl	CO_2 cysHCl
E. stiedai (intact)	34.511	0.00116	0.00119	0.00434	0.00706
(NaOCl-treated)	34.511	0.00020	0.00168	0.00204	0.00560
E. tenella (intact)	22.025	0.00759	0.00844	0.00809	0.00944
(NaOCl-treated)	22.025	0.00644	0.00809	0.00809	0.00844

to reach maximum activation. After incubation, oocyst suspensions were washed 5 times with distilled water before being titrated for sulfhydryl (–SH) groups.

Sulfhydryl group determinations were made on each oocyst suspension within 4 hr of its incubation. Fifteen or 30 μliters of 3, 3'-dithiobis-6-nitrobenzoate (DTNB, Eastman Kodak Co.) at 0.005 M in ethanol were added to the washed oocyst suspensions and each was thoroughly mixed with a vortex stirrer. Three milliliters of tris-HCl were slowly added to the suspension while mixing. The volumes were finally brought to 5 ml with the buffer. The DTNB reacted with the –SH groups at pH 7.3 to 7.5. The suspension was then centrifuged, and the supernatant was filtered through a 0.45-μm Millipore filter before being examined for optical density at 412 mμ. An estimate of the –SH group content of each test suspension was made by comparing test results with a standard curve prepared with known concentrations of cysteine. Values stated are based on an average resulting from 3 replications.

RESULTS

The treatment of *E. stiedai* with cysHCl under CO_2 produced more –SH groups than the three control treatments in intact as well as NaOCl-stripped oocysts of both species (Table I). The next highest to lowest in order were the cysHCl under air, water under CO_2, and water under air treatments, respectively. With the single exception of the oocysts treated in water under CO_2, intact oocysts contained higher quantities of –SH groups than corresponding treatment groups of stripped oocysts. Excystation tests of subsamples of each incubation indicated activation levels of >95% (cysHCl under CO_2), <3% (cysHCl under air), <1% (water under CO_2), and <1% (water under air) before –SH titrations were run.

Activation of sporozoites with trypsin and bile never exceeded 1% in intact *E. tenella* oocyst suspensions before the washing and testing procedures were run, whereas that of the NaOCl-stripped oocysts reached a level of approximately 60% when incubated in cysHCl under CO_2. None of the control groups exceeded 0.5% activation during the periods of incubation. As with *E. stiedai*, the cysHCl under CO_2 treatment produced the highest numbers of –SH groups of the four treatments (Table I). The intact oocysts incubated in cysHCl under air yielded fewer –SH groups than those in water under CO_2 but more than those in water under air. No difference in –SH group level was apparent in stripped oocysts incubated in cysHCl under air or in water under CO_2. The lowest level in stripped *E. tenella* oocysts occurred in those incubated in water under air. Generally, more –SH groups were found in intact oocyst groups than in stripped of outer walls, and the levels in *E. tenella* were higher than those in *E. stiedai*.

DISCUSSION

The DTNB test indicated that decreases in –SH groups occurred in the oocyst walls during treatment with cysHCl and CO_2. This change was coincident with gross structural changes seen microscopically as wall warpage and/or micropylar opening in intact and NaOCl-stripped oocysts of *E. stiedai* and in stripped oocysts of *E. tenella*. The visual warpage was not apparent in intact *E. tenella* oocysts although the –SH group increase was readily detected with the spectrophotometer. Apparently, the reducing agent $-CO_2$ combination reduced disulfide bonds stabilizing the protein structure of the walls. By comparing –SH group quantities found in intact oocysts with those in stripped oocysts we found that much of the change occurred in the outer layer of the walls. Outer layers of oocysts of several species of *Eimeria* and *Isopora* are composed of quinone-tanned protein (Monné and Hönig, 1954). Ryley (1973) found the outer shell of *E. tenella* to consist mainly of protein with a high proline content and low basic amino acid content. Our results suggest that cysteine is present, an
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that cysteine is present, and probably aids in maintaining oocyst wall integrity.

The inner layer of *E. tenella* oocyst walls was determined by Ryley (1973) to be protein of normal amino acid content, lipid, and a small amount of carbohydrate. The inner layers of the oocysts studied by Monné and Hönig (1954) were found to be a protein matrix impregnated with lipid, with the highest lipid content residing in a zone near the oocyst cavity. In our studies neither distilled water under air, distilled water under CO_2, nor cysHCl under air were effective in altering oocyst walls to the extent that we could attain appreciable sporozoite activation with trypsin and bile. The lipid in the inner wall layers apparently protects structural proteins against the action of these agents as well as most chemicals. Ryley (1973) found intact as well as stripped oocysts resistant to trypsin, pepsin, and pronase only until the oocysts were fractured. We found the combination of cysHCl and CO_2 to effectively alter the outer as well as the inner wall layer and make all but a thin membrane of the innermost portion susceptible to degradation by NaOCl.

Jackson (1962), Hibbert and Hammond (1968), and Ryley (1973) suggested that an oocystic or sporocystic enzyme could be mediating the primary (wall altering) phase of excystation after being activated by CO_2. Such an enzyme has not been identified, is not located in a soluble portion of the oocyst, and, if present, is not active when applied to the outside of viable oocysts (Jolley and Nyberg, 1974). Furthermore, if such an enzyme is present, it is relatively nonspecific in its need for an activator, since oocysts of *Eimeria bovis*, *E. stiedai*, and *E. tenella* were altered by substituting hydrogen sulfide (H_2S) or nitric oxide (NO) for CO_2 in the excystation procedure (Jensen et al., 1976). Chemically, H_2S and NO more nearly resemble the action of reducing agents than of CO_2.

Neither CO_2 nor the reducing agents alone are effective in altering the oocyst walls, although Jensen et al. (1976) were able to cause an appreciable change in the permeability of *E. stiedai* oocysts by flushing H_2S over a shallow suspension of oocysts in distilled water. This recent evidence indicates that the reducing agent in the CO_2-reducing agent complex may play a more important role in in vitro excystation than was formerly recognized. As suggested by Jolley and Nyberg (1974), the complex formed in the in vitro incubation medium between CO_2 and the reducing agent may provide the proper configuration for unmasking and reducing the disulfide bridges in the oocyst wall which are critical in maintaining the relatively impermeable state of intact oocysts. Although the complex has not been characterized, it is probably a common reaction between CO_2 and other reducing agents, since, with the exception of H_2S (Jensen et al., 1976), none has been reported to be active alone but become active when combined with the CO_2, e.g., sodium dithionite can be substituted for cysHCl in the excystation procedure.

The finding of –SH groups in oocysts within the various treatments described in this study indicates an accessibility of many disulfide bridges in areas of the oocyst walls which are not crucial to oocyst permeability. These groups are probably in the outer layers of the oocysts. When the synergistic combination of CO_2 and cysHCl are present, the key structural stabilizers in the inner wall layers may be broken. We visualize the effect of the complex as being similar to the simple "lock and key" enzyme arrangement whereby the CO_2-cysHCl complex matches two specific sites in the oocyst wall, one of which is a lipid "mask" and the other a masked disulfide bond. As the disulfide reduction proceeds, wall alteration is then compounded by the stresses of progressive cracking, twisting, and folding. In an EM study of in vitro excystation, to be reported elsewhere, a uniform, ringlike area encircles the micropylar "trapdoor" in *E. stiedai* oocysts. As the micropylar area appears to sink inwardly, the outer wall layer around the depression cracks and curls, and the "lid" finally curls or folds inwardly, leaving a very uniform opening which is more or less clear of obstruction. Many of these features are also seen in *E. tenella* oocysts which have been stripped of the outer wall layers by NaOCl and treated with CO_2 and the reducing medium, although not as many open completely as do those of *E. stiedai*. The ringlike area around the micropylar "lid" of sporulated oocysts probably contains a relatively high number of vulnerable disulfide bridges.

LITERATURE CITED

HIBBERT, L. F., AND D. M. HAMMOND. 1968. Effects of temperature on excystation of various *Eimeria* species. Exp Parasitol 23: 161–170.

JACKSON, A. R. B. 1962. Excystation of *Eimeria arlwingi* (Marotel, 1905): Stimuli from the host sheep. Nature 194: 847–849.

JENSEN, J. B., P. A. NYBERG, S. D. BURTON, AND W. R. JOLLEY. 1976. The effects of selected gases on excystation of coccidian oocysts. J Parasitol 62: 195–198.

JOLLEY, W. R., AND P. A. NYBERG. 1974. Formation of a carbon dioxide–cysteine complex in the incubation fluid used for excysting *Eimeria* species in vitro. Proc Helminthol Soc Wash 41: 259–260.

MONNÉ, L., AND G. HÖNIG. 1954. On the properties of the shells of the coccidian oocysts. Ark Zool 7: 251–256.

NYBERG, P. A., D. H. BAUER, AND S. E. KNAPP. 1968. Carbon dioxide as the initial stimulus for excystation of *Eimeria tenella* oocysts. J Protozol 15: 144–148.

RYLEY, J. F. 1973. In the Coccidia. D. M. Hammond with P. L. Long (eds). Univ. Park Press, Baltimore, Butterworths, and London, p. 151–166.

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James B. Jensen* and S.
Department of Poultry Scien
Auburn, Alabama 36830

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